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Full Length Research Paper

In vivo evaluation on Malaysian coastal isolates of *Gracilaria changii* and *Stichopus badionotus* through heat-burn methicillin-resistant *Staphylococcus aureus* (MRSA) infection animal model

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Staphylococcus aureus **commonly causes bacterial infections such as boils, carbuncles, infections wounds, deep abscesses and bloodstream infections (or bacteraemia). Nowadays, it becomes more difficult to treat such infections due to the appearance of resistant strain known as methicillin-resistant** ***Staphylococcus aureus*** **(MRSA). The objective of this study was to screen on methanolic extracts of seaweed (*Gracilaria changii*) and local sea cucumber (*Stichopus badionotus)* for their anti-MRSA activities *in vitro* and *in vivo;* by a heat-burn, wound-healing model in rats. Both extracts demonstrated *in vitro* anti-MRSA activity. The sea cucumber extract demonstrated the same extent of wound healing as did vancomycin at P<0.05. The wound-healing activity of the seaweed extract was superior to that of vancomycin as shown by Tukey's test (P<0.05). It was concluded that both of these crude extracts have a potential to become an alternative resource for anti-MRSA drugs.**

Key words: *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), heat-burn injury, seaweed, sea cucumber, Malaysia.

INTRODUCTION

Due to the emergence of methicillin-resistant strains of *Staphylococcus aureus,* there is need for development of alternative antimicrobial compounds. Although, there are more than 150 000 macro-algae or seaweed species found in the oceans of the globe, their production of antibiotics has not been fully assessed. These organisms which produce primary and secondary metabolites may be potential bioactive compounds of interest for the pharmacological industry since special attention has been reported for antiviral, antibacterial and antifungal activities related to the application of marine algae against several pathogens (Lima-Filho et al., 2002).

Gracilaria changii that belong to the *Gracelariaceae* family is red seaweed that grows abundantly in muddy, silted areas, especially in the mangroves fringing the west coast of peninsular Malaysia. The Gracilaria genus in general, is usually studied for higher yield of agar production (Bezerra et al., 2010). However, the production of red algae as an antibacterial agent has not been studied in detail, especially those from Malaysian coastline. Many bioactive and pharmacologically active substances have been isolated from algae (Singh et al., 2005; Plaza et al., 2010). In recent studies, it has been shown that *G. changii* has an antifungal activity that is active against *Candida albicans* (Screenivasan et al., 2009). The other marine organism; *Stichopus badionotus* sluiter (In Malay words known as Gamat) has antimicrobial activity against methicillin-resistant strain of *S. aureus* (MRSA) as described previously by Mariana et al. (2009).

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MATERIALS AND METHODS

Source of the marine extracts

The marine organisms used in this study were *G. changii (G. changii)* and sea cucumber (*S. badionotus*). A dried specimen of *G. changii* was supplied by Dr. Habsah Mohamad from Universiti Malaysia Terengganu (UMT), Malaysia and a sea cucumber specimen (without internal organs) was supplied by Laboratory of Marine Science and Aquaculture, Institute of Bioscience, Universiti Putra Malaysia. A dried specimen of both organisms was grinded into smaller pieces to increase the surface area prior to extraction protocols. They were soaked overnight in absolute methanol under mild agitation (80 rpm) and the specimens were filtered through Whatman® (no.1) filter paper. The remaining of methanol in the recovered liquid was removed by rotary evaporator and the recovered specimen-gum was diluted with 10% methanol. The extracts were stored at 4°C until further use.

Bacterial strain

Methicillin resistant *S. aureus* (MRSA) references strain was used; ATCC700698 and was obtained from the stock culture of Microbiology Research Laboratory, Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

Antimicrobial activity of the crude extracts

Both of the extracts showed an inhibition zone against *S. aureus*, and were tested on both methicillin resistant and sensitive strains in Kirby-Bauer test as described previously by Mariana et al. (2009). The minimal inhibitory concentration was successfully observed for both extracts and these concentrations were use as a treatment concentration on animal model. The selection of the dosage was based on our previous study (Mariana et al., 2009).

Animal studies

Male Sprague Dawley rats aged 3 months with similar weight and size were used in this study. All the subjects were in good health. The animals were housed in individual cages prior to this study and received standard pellet diet and clean water as daily diet. All animals were acclimated to the cage conditions and diet for 1 week before the treatment started. These animals were divided into groups of 5 for each treatment. After the wounds were introduced, the best 3 animal which developed with minimal wound size differences were group together as treatment group. The exclusion was made to minimize the wound size differences as a starting measurement point.

Induction and measurement of the heat burn injury infection model

The ethical approval was obtained from the Animal Care and Use Committee (ACUC), Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. The heat-burn wound model was performed as described previously by Knabl (1999). The wounded site was observed daily after bacterial inoculation. The formations of red, with possible pus formation at the wound area were considered as successful skin infection. For further confirmation, the excretion at the wounded site was observed for the pus cells and also the possible bacterial cells under microscopic observation. Routine bacterial culture on blood agar plate were made to observe

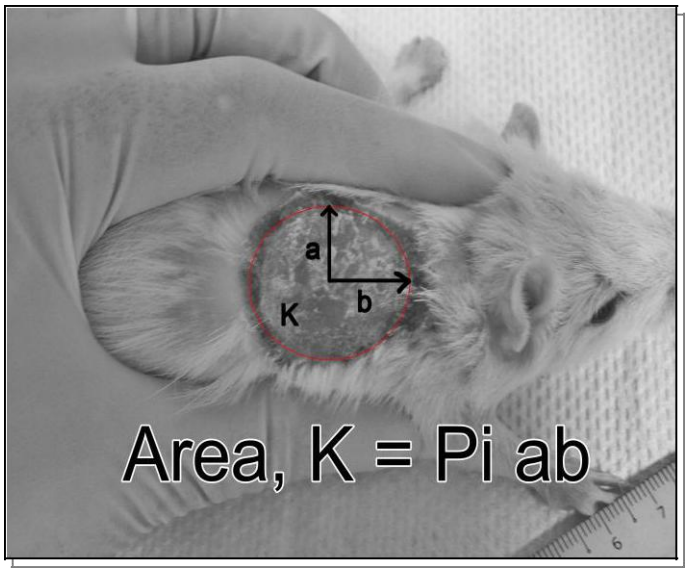

Figure 1. Measurement of the wound area. The wound area is expressed in cm^2 as a result of multiplication of *a* with *b* value. To minimize the wound size differences as starting area, animals with similar wound area measurement were grouped into same treatment group.

the presence of *S. aureus* and was further tested in antibiogram pattern to re-confirm the presence of MRSA from the wounded site.

Topical skin treatment with the extracts

The treatment was made 24 h after successful skin infection. The concentration of the extracts and the controls used at the volume of 200 µl per treatment in this study were; 30 µg/ml sea cucumber, 18 µg/ml seaweed, 5% w/v vancomycin, 5% w/v penicillin and 40% v/v methanol as a diluents control. The solution was applied on the surface at the site of the infection by using micropipette and left to air dry. The treatment was made twice daily, until the wounded site was successfully healed.

Measurement of wound area

The area of the wound was measured daily for the first 10 days of treatment and later every 2 days until day 30. The measurement was made using a ruler and was measured in centimeter unit. The formula used in the measurement of wound area is Area (K) = Pi ab as illustrated in Figure 1. The wound size differences were measured by the measurement of day_1 to day_0, which represent the wound size differences in between point of measured value. The wound size difference values from day 1 until day 30 were tabulated, and the wound size decrement and increment can be observed by the plotted graph.

Statistical analysis

All statistical analysis was performed by using SPSS software (Version 11.0, SPSS Inc., Chicago, IL, USA). Results of wound size reduction were expressed as mean±SD. Interaction of treatment efficacy between groups were performed by one-way analysis of variance and further Post-Hoc Tukey's test. The acceptance level of significance was $P < 0.05$.

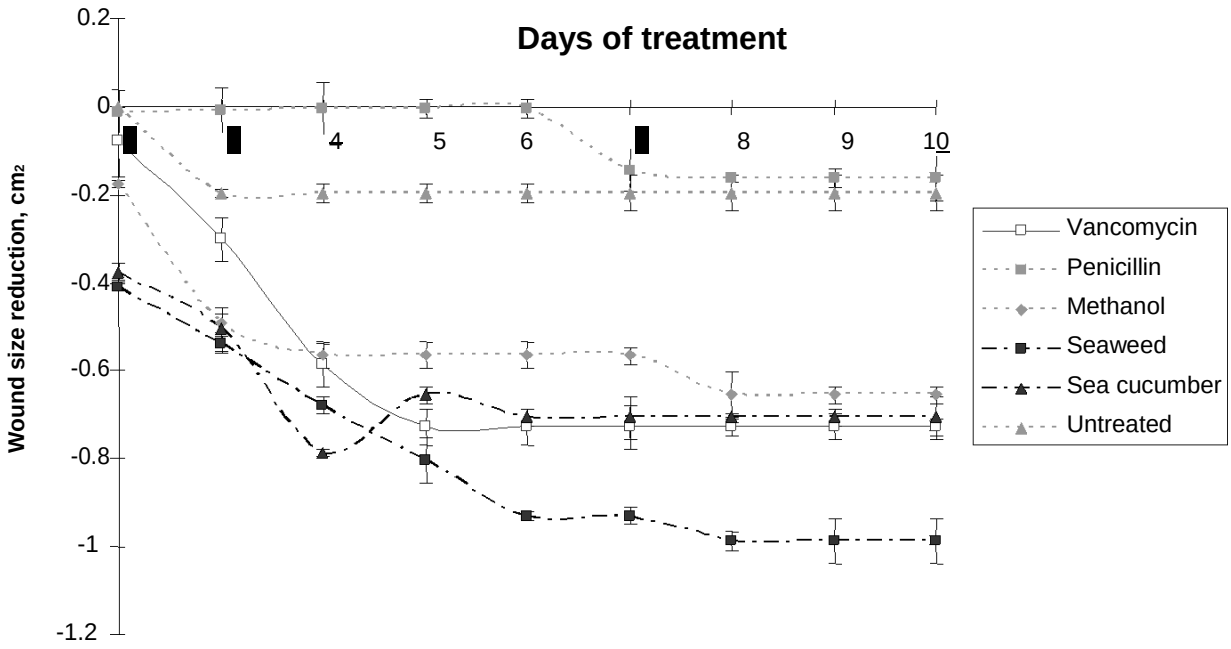

Figure 2. Wound area reduction on first 10 days of treatment of infected subjects with MRSA. Each point represents a mean value taken from 3 animals. Wound size area is expressed in differences value on current day to day 0 (Day_1 - Day_0) and represent in mean ± SD (n = 3 animals per group). $P < 0.05$, the wound reduction measurement of sea cucumber was calculated with no significant different with vancomycin. As for seaweed, the effect was at significant different with better reduction rate as compared with vancomycin.

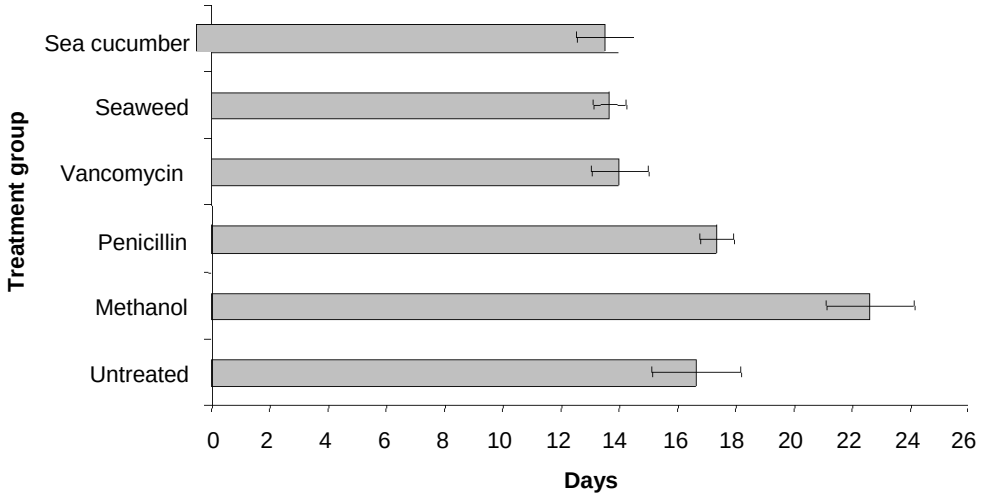

Figure 3. Time of complete detachment of wound's scab prior to treatment. The days represent in mean±SD (n = 3 animals) of time that wound's scab was fully detached from its site. The finding suggests that the treatment group of sea cucumber, seaweed and vancomycin produced a better healing, with time towards the infected wounded site.

RESULTS

The graph of wound size reduction was plotted by using the value of the wound size differences with the mean value of group measurement with first day of treatment. Figure 2 showed the comparative effect of the extracts and several controls against MRSA, based on wound size reduction from day 1 until day 10. The further observation on wound showed that the treatment with both extracts produced a better outcome with a faster detachment of skin's scar on wounded site as shown in Figure 3.

In addition, the newly produced skin under the wounded area was also more dry and clean as compared to control group. In the measurement of bacterial viability, general platting technique showed that the recovered excretion from the wounded site contained *S. aureus* and mixed with other bacterial; probably from environment. Microscopic examination of the excretion showed pus cells, together with cocci-shaped bacteria in which represent the cocci in shape *S. aureus.* Recovered bacterial colonies from streaking technique and also the coagulase positive isolate then further analysed for their antibiogram against ß-lactam group antibiotics; penicllin, oxacillin and cefoxitin. The antibiogram showed a level of resistance when compared with the table of guideline provided by Clinical and Laboratory Standard Institute, CLSI 2007 which re-confirmed the recovery of MRSA from the wounded site.

DISCUSSION

The results were influenced by the fact that not all the rats were equally restrained and restraining procedures have been shown to stress the animal and slow the healing progress (Sheridan et al., 2004). It was observed that the group that received methanol and penicillin as treatment takes a longer time to fully detach the skin's scars from the wounded site, as compared to untreated group that did not receive any restraining procedure until the end of study. Since the burn was induced at the animal's dorsal part, self-scratching by the animals were reduced as the wounded area was not accessible by the animal's hind and front legs.

However, it was observed that a few of the test subjects were active and aggressively laid their dorsal part on the cage's metal fence and also on bedding materials. These activities will force the early detachment of the scab from the wounded site and this can be observed by the ripening-red (and bleeding) under the scab area which forcefully detached. In these experi-ments, the methanolic extracts were applied. Increased rates of wound size reduction might be anticipated, if the extracts were applied in an ointment or cream. Oil base or cream will retain the treatment time as the crude extracts used in this study were water base prepared. Further purification of the methanolic extracts is needed, since the exact component that responsible for antimicro-bial and wound healing properties is not identified through this study. The initial study has showed that the compound that is present in the crude methanolic extracts of sea cucumber has a property inhibiting the formation of bacterial cell membrane. However, the rapid and extensive effect of the bioactive particles which are present in both extracts are currently being studied by the same group of researchers including the synergistic effect of extract with commercial antibiotics. Sulfated fucans which have been widely isolated from the body wall of sea cucumber, including the *Stichopus* spp. has been shown to have the abilities of a carrier for few antibiotics against *S. aureus* (Araújo et al., 2004; Caldwell et al., 2010). This ability might be the key for a better drug immobilization in the synergistic study of drug treatment in combination.

Conclusion

Methanolic extracts of *G. changii* and *S. bodionotus* were able to accelerate healing in a heat-wounded, MRSA or *S. aureus*-infected rats. A further investigation is needed to completely utilize the full potential of Malaysian local resources as an alternative in drugs development.

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